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Review

FAN1, a DNA Repair Nuclease, as a Modifier of Repeat Expansion Disorders

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Abstract. *FAN1* encodes a DNA repair nuclease. Genetic deficiencies, copy number variants, and single nucleotide variants of *FAN1* have been linked to karyomegalic interstitial nephritis, 15q13.3 microdeletion/microduplication syndrome (autism, schizophrenia, and epilepsy), cancer, and most recently repeat expansion diseases. For seven CAG repeat expansion diseases (Huntington's disease (HD) and certain spinocerebellar ataxias), modification of age of onset is linked to variants of specific DNA repair proteins. *FAN1* variants are the strongest modifiers. Non-coding disease-delaying *FAN1* variants and coding disease-hastening variants (p.R507H and p.R377W) are known, where the former may lead to increased FAN1 levels and the latter have unknown effects upon FAN1 functions. Current thoughts are that ongoing repeat expansions in disease-vulnerable tissues, as individuals age, promote disease onset. Fan1 is required to suppress against high levels of ongoing somatic CAG and CGG repeat expansions in tissues of HD and *FMR1* transgenic mice respectively, in addition to participating in DNA interstrand crosslink repair. *FAN1* is also a modifier of autism, schizophrenia, and epilepsy. Coupled with the association of these diseases with repeat expansions, this suggests a common mechanism, by which FAN1 modifies repeat diseases. Yet how any of the *FAN1* variants modify disease is unknown. Here, we review *FAN1* variants, associated clinical effects, protein structure, and the enzyme's attributed functional roles. We highlight how variants may alter its activities in DNA damage response and/or repeat instability. A thorough awareness of the *FAN1* gene and FAN1 protein functions will reveal if and how it may be targeted for clinical benefit.

Keywords: DNA repair, FAN1, Huntington's disease, karyomegalic interstitial nephritis, modifier, nuclease, repeat instability

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Genome integrity is maintained by the concerted action of distinct DNA repair pathways. DNA nucleases resolve damaged DNA and unusual DNA structures that arise during cellular processes such as replication, recombination, and transcription. DNA repair nucleases usually recognize aberrant DNA conformations of damaged and/or potentially mutagenic DNA intermediates, rather than DNA sequences; hence, they are referred to as ‘structure-specific nucleases’. DNA repair machinery corrects errors incurred during replication in mitotic cells, and repairs damaged DNA in non-mitotic cells [1]. Pro-

cessing of aberrant DNA structures, particularly steps involving nucleolytic cleavage, represents a committed and tightly controlled biological ‘decision’ in genome maintenance pathways. If not kept in check, structure-specific nucleases can cause extensive DNA degradation and wreak havoc on the stability of the genome [2]. On the other hand, the essential nature of structure-specific nucleases is underscored by the fact that homozygous null mutations in genes encoding these enzymes are often embryonic lethal or lead to infertility; single site mutations may predispose to cancer or other diseases (for review, see [2]). This review focuses on *FAN1*, with particular attention to its potential roles as a modifier of disorders associated with genomic repeat expansions, including Huntington’s disease (HD), spinocerebellar ataxia type 1 (SCA1), SCA2, SCA3, SCA6, SCA7, SCA17, autism, schizophrenia, and others.

1. *FAN1* GENIC REGION AND DISEASE-ASSOCIATED GENETIC VARIANTS

1.1. *Genes*

FAN1 (OMIM#613534) was initially identified as *KIAA1018* in a human brain cDNA library and found to be ubiquitously expressed in the central nervous system and peripheral tissues [3]. The protein encoded by *KIAA1018* interacts with the mismatch repair (MMR) proteins, PMS2 [4] and MLH1 [4], and *FANCD2* [5–8]. Except where noted, we refer to the gene in question as *FAN1*. *FAN1*, at 15q13.3 (GRCh37/hg19 coordinates: chr15:31,196,076-31,235,31) contains 15 exons, of which 13 are coding. The gene’s *cis*-acting regions include promoter regions, CpG islands, transcription factor binding sites, and enhancers (Fig. 1b). It has three predicted promoters (as per EPD, ENSEMBL, and NCBI), two of which are located upstream of exon 1, while the third promoter resides in intron 8. A large CpG island of 918 base pairs (bp) spans exon 1, promoter 1 and further upstream [9–11] (Fig. 1b). Various *FAN1* variants and the *FAN1* haplotypes

Fig. 1. Genomic region of human *FAN1* at 15q13.3 (GRCh37/hg19). (a) Schematic organization of 15q13.3 microduplication/microdeletion region contains multiple genes including *FAN1*. (b) *FAN1* and *MTMR10* are partially overlapped and include various coding and non-coding genetic variants identified in HD, various spinocerebellar ataxias (SCAs), autism (ASD), and schizophrenia (SZC). The murine homolog of p.S795P (mS798P, in between the TPR and the nuclease domain) was identified in a murine Rett syndrome modifier screen [86]. CAG/polyQ disease-hastening and disease-delaying variants are shown in red and green fonts, respectively. (c) *FAN1* has various transcript variants, a limited set of full length and truncated *FAN1* transcript variants are shown (NM: Transcript variant, NP: Isoforms of transcript variant). Information for making this figure is from the NCBI genome browser, UCSC genome browser, and Ensembl browser. For details of the 15q13.3 chromosomal rearrangements, see Supplementary Figure 1. (d) Table with HD modifier haplotypes in *FAN1*.

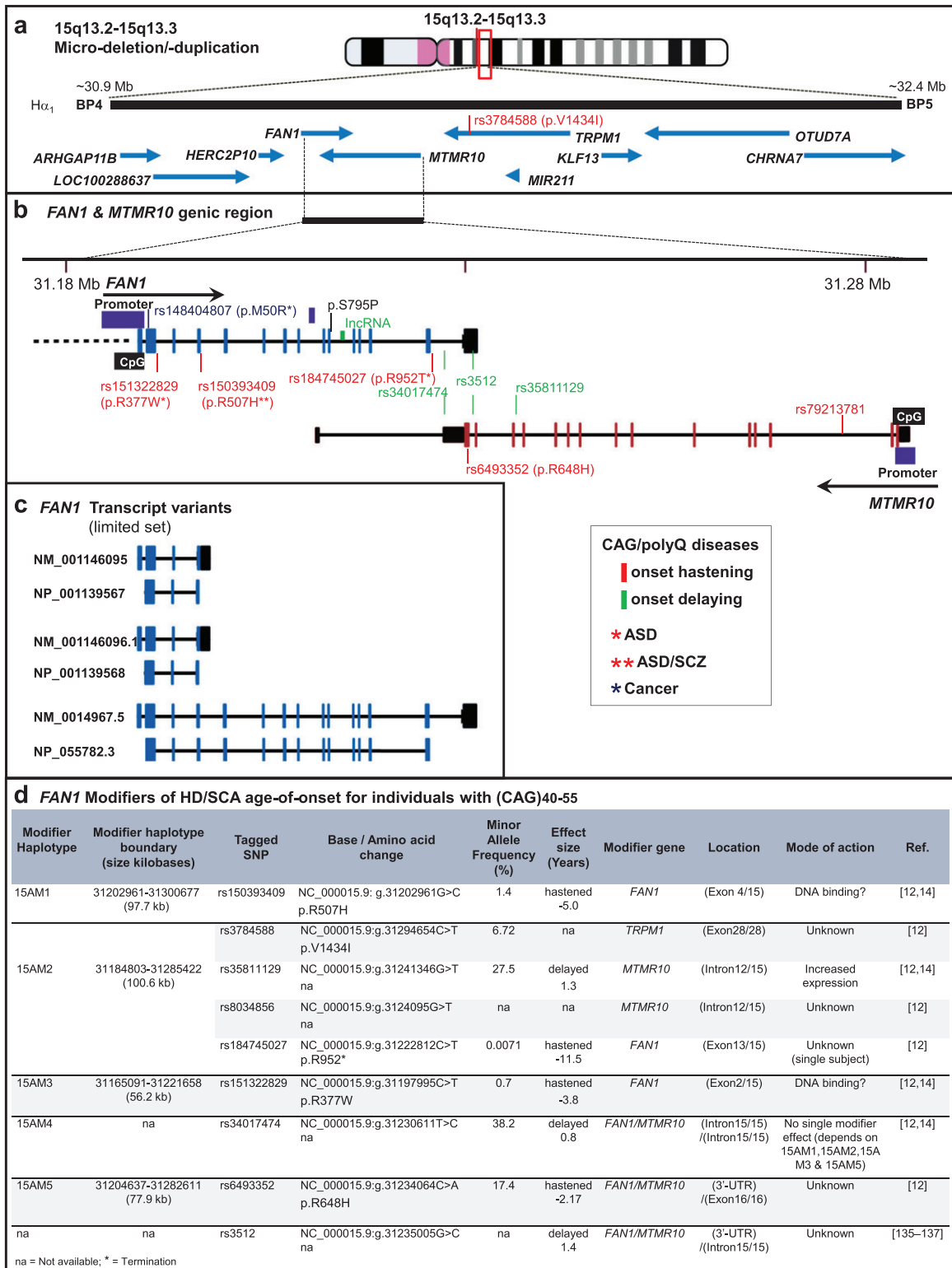


Fig. 1. (Continued)

associated with HD and SCAs are shown in Fig. 1 (summarized in 1d) and discussed in detail in Section 6.1 and 6.2.

1.2. Transcripts

FAN1 transcripts are predicted to undergo alternative splicing, generating 28 potential transcript variants (Fig. 1c), which code for full-length or truncated proteins. Some transcripts are predicted to undergo nonsense-mediated decay, and some retain introns [11]. A *FAN1* splicing effect on HD modifiers is unlikely, based upon analyses of data in non-disease expression databases [12]. *FAN1/Fan1* circular RNA (circRNA) was identified in human and mouse heart, but is of unknown function [13]. The *MTMR10* gene partially overlaps with *FAN1* and is convergently transcribed (Fig. 1a,b). Disease onset-modifying variants in overlapping regions of these two genes have been identified (Fig. 1). *MTMR10*-tagging single nucleotide polymorphisms (SNPs) identified in individuals with HD or SCA2 may relate to *FAN1* expression levels [14–16]. *MTMR10* transcripts could hypothetically act in an antisense manner to regulate *FAN1* mRNA stability and translatability. The same might be true where a *FAN1* transcript regulates the *MTMR10* mRNA. Evidence of such antisense regulation is not known for these genes but could provide insight into targeting for therapeutic purposes.

1.3. *MTMR10* and *TRPM1*

The 15q13.3 region with adjacent genes, *FAN1*, *MTMR10*, and *TRPM1* (Transient Receptor Potential Melastatin Family member 1) (Fig. 1a), appears to be enriched with variants that modify HD onset. *FAN1* has been referred to as *MTMR15* [17]; however no data reveal any relation of *FAN1* to myotubularin-related proteins (MTMs) [7]. In contrast, *MTMR10* is an MTMR family member. Various tagSNPs associated with HD age of onset are within or proximal to the two overlapping convergently transcribed genes, *MTMR10* and *FAN1* (Fig. 1b,d). A protein coding variant in *TRPM1* (rs3784588; p.V1434I) is associated with hastening HD onset [12]. The roles of *MTMR10* or *TRPM1*, if any, in HD or any of the 15q13.3-related diseases, are unknown. *TRPM1* and *MTMR10* have been argued to not be HD modifiers, based upon genetic data [14] and the absence of an effect of the disease-delaying variant haplotype (15AM2) upon expression levels of either *TRPM1* or *MTMR10* in non-HD databases [12, 14]. More-

over, genetic ablation of *Mtmr10* does not affect CAG instability in HD mice, whereas ablation of *Fan1* does [18]. The effect of *TRPM1/Trpm1* on CAG instability in human or mouse models has not been addressed.

1.4. 15q13.3 micro-deletion/-duplication, and CNVs

Genetic variants of *FAN1* include copy number variations (CNVs), inversions, and single nucleotide variants (SNVs). *FAN1* and *MTMR10* lie within a 2 Mb region, 15q13.3, which can present copy number variation of zero, one, two, three, or four copies [19, 20]. This 15q13.3 region contains at least seven genes [21–23], with at least five distinct human-specific structural configurations, including a common inversion [24] (Fig. 1 and Supplementary Figure 1). The genes include *ARHGAP11B*, *FAN1*, *MTMR10*, *TRPM1*, *miR211*, *KLF13*, *OTUD7A*, and *CHRNA7* (Fig. 1). Microdeletions of 15q13.3 can be homozygous, heterozygous or compound heterozygous. Homozygous and compound heterozygous 15q13.3 microdeletions are associated with severe phenotypes, which may include intellectual disability, dysmorphic features, epilepsy, neuropsychiatric disturbances with or without cognitive impairment, schizophrenia and autism [21, 25]. In contrast, individuals with heterozygous 15q13.3 microdeletions may show a mild phenotype or complete absence of anomalies [21–23]. Duplications of 15q13.3 are less commonly observed than deletions, and less often associated with intellectual disability [19, 20]. CNVs of the 15q13.3 region could increase or decrease the levels of the *FAN1*, *MTMR10*, and *TRPM1* gene products, which may affect phenotypes. However, the mechanism by which CNVs of any of these genes relate to clinical presentations remains unclear. Of the various genomic configurations of the 15q13.3 region (Supplementary Figure 1), it is not known which occur with the HD-modifying *FAN1* and *MTMR10* variants. It would be interesting to learn whether some 15q13.3 structural configurations are associated with modifying the presentation of HD, SCAs, autism, or schizophrenia.

2. *FAN1* PROTEIN STRUCTURE, DOMAINS, AND PROTEIN INTERACTIONS

In 2010, four groups independently identified *KIAA1018* as a structure-specific nuclease required for DNA interstrand crosslink (ICL) repair; they collectively renamed the protein *FAN1* (for *FANCD2*-

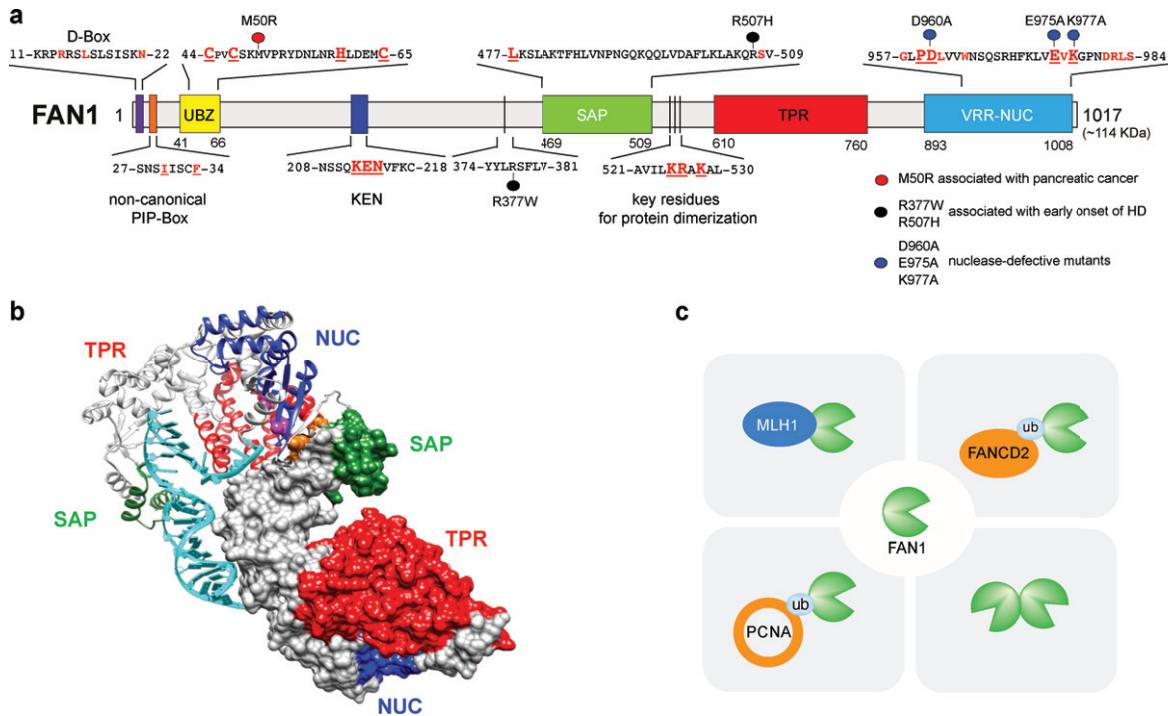


Fig. 2. Protein domains of FAN1. (a) FAN1 is composed of an unstructured N-terminal region containing the Ubiquitin Binding Zinc (UBZ) finger domain, a central region containing a SAP domain to mediate DNA binding and a TPR domain required for protein-protein interactions, and a C-terminal region embedding a VRR_NUC domain that harbors the catalytic site. A non-canonical PCNA-interacting peptide (PIP) motif and a KEN-box motif, which are required for interaction with PCNA and Cdh1, respectively, are located at the N-terminal region. Key residues of each domain are highlighted in red. Mutations associated with pancreatic cancer and early onset of HD are highlighted with red and black circles, respectively. (b) Crystal structure of FAN1 revealed that two FAN1 molecules can dimerize to form a head-to-tail dimer in the presence of DNA. Assembly of FAN1 dimer occurs via the TPR and NUC domains of the first FAN1 molecule interacting with the SAP domain of a second FAN1 [41]. (c) FAN1 can form different subcomplexes with MLH1, ubiquitylated FANCD2, ubiquitylated PCNA, or can exist as homodimer in solution.

FANCI-associated nuclease 1) due to its physical association with FANCD2—a central component of the Fanconi Anemia (FA) pathway [5–8]. The fundamental importance of FAN1 in cell physiology is underscored by its domain organization, which is highly conserved among eukaryotes and also present in some bacteria [5–8] (Fig. 2a, b). All FAN1 orthologs contain a C-terminal PDX_n(D/E)XK motif located within an atypical virus-type replication-repair nuclease (VRR_NUC) domain [26, 27]. FAN1 is the only known eukaryotic nuclease with a VRR_NUC module, which is otherwise common in bacteriophages and bacteria as a stand-alone domain. FAN1 preferentially incises 5'-flap branched structures [5–7, 28] (Fig. 3). However, FAN1's substrate specificity appears to be broad; its endonuclease activity can efficiently cleave different DNA intermediates and probably deal with several types of DNA lesions. Besides its ability to cleave DNA substrates containing a 5'-flap, FAN1 displays a 5'-3'

exo-nuclease activity that requires gapped, nicked, or recessed DNA as an entry point [5–7] (Fig. 3) and can cleave every 3rd nucleotide in small flap non-repeat DNA [29]. FAN1 can also cleave D-loop structures that form during homologous recombination repair (HRR) [6] (Fig. 3). DNA damage induced by exogenous or endogenous chemicals, including ICLs and oxidative DNA lesions, have also been considered as targets of FAN1 (detailed in Section 4).

FAN1's N-terminal region is largely unstructured, except for a RAD18-like ubiquitin-binding zinc finger (UBZ) domain, which is required for FAN1 localization to ICLs and stalled replication forks, possibly through binding to ubiquitylated FANCD2 [5–8, 30]. Consistently, a FAN1 zinc-finger mutant (C44A/C47A, Fig. 2a) fails to co-localize with FANCD2 in DNA damage-induced nuclear foci [5–8]. However, the UBZ mutant is competent in rescuing from mitomycin C (MMC) sensitivity of FAN1 knockout cells, suggesting that ubiquitin

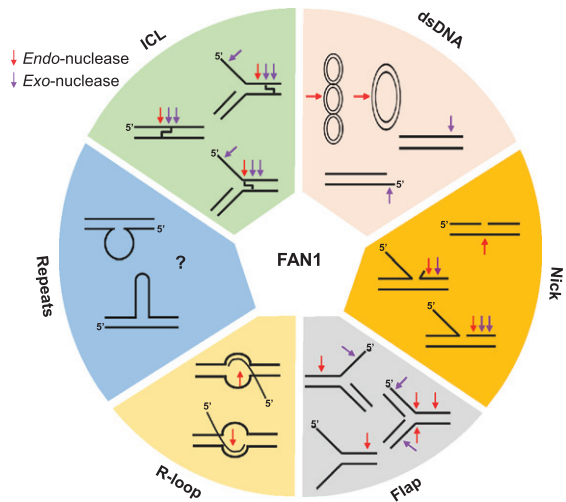


Fig. 3. FAN1 can process various unusual DNA structures, including interstrand cross-linked DNA (ICL), dsDNA (supercoiled, covalently closed circular, linear DNA), nicked DNA, flap-DNA, R-loop DNA, and repeat containing DNA (unknown). Purple and red arrows denote *exo*-nuclease and *endo*-nucleolytic cleavage, respectively.

recognition is not essential for ICL repair [30–33]. In agreement with this, the FAN1 p.M50R cancer-associated mutant—located in the UBZ domain (Fig. 2a)—equally abolishes FAN1 foci but restores MMC resistance in FAN1-deficient cells [30], indicating that other FAN1 domains can mediate recruitment to ICLs. Indeed, FAN1 harbours a non-canonical proliferating cell nuclear antigen (PCNA)-interacting peptide (PIP) box motif that precedes and acts in concert with the UBZ domain in binding to ubiquitylated PCNA (ub-PCNA) [34] (Fig. 2a). A systematic and comparative analysis measuring the recruitment kinetics of DNA repair proteins at complex DNA lesions indicated two separate modes of FAN1 recruitment: an early step clustering with PCNA and a later step overlapping with FANCD2 [35]. Moreover, the PIP- and UBZ-dependent interaction of FAN1 with ubiquitylated PCNA is reminiscent of that for DNA polymerase η , acting in translesion DNA synthesis (TLS) [36–38], and for Spartan, a metallo-protease acting in DNA-protein crosslink repair [39]. Remarkably, both the PIP box and the UBZ are well conserved in FAN1 of multicellular eukaryotes including *C. elegans*, but absent in *S. pombe*, suggesting an evolutionary expansion of FAN1 interaction networks.

FAN1 contains highly conserved SAP (SAF-A/B, Acinus and PIAS) and TPR (tetratricopeptide repeat) domains implicated in DNA binding and FAN1 dimerization [28, 40] (Fig. 2a, b). FAN1 is monomeric in solution, but dimerizes in the presence

of DNA [28]. Human FAN1 forms an asymmetrical ‘head-to-tail’ FAN1 dimer on DNA (Fig. 2b) [41]. Dimerization relies on a region of the SAP domain of one FAN1 molecule interacting with the TPR and NUC domains of a second FAN1 molecule [41]. The first SAP domain defines the DNA substrate orientation, whereas the TPR and NUC domains of the second FAN1 facilitate the nucleolytic reaction by engaging the cognate DNA in the catalytic site [41] (Fig. 2b). While FAN1 dimerization endows the protein with its specific *endo*-nuclease activity, dimerization is not absolutely required for its *exo*-nucleolytic function [41]. The FAN1 dimer-DNA crystal structure displays three different DNA binding modes (‘substrate-scanning’, ‘substrate-latching’, and ‘substrate-unwinding’), which determine distinct mechanisms for DNA cleavage [41]. Along this line, FAN1 homodimers process long (40 bp) but not short flap (1–5 bp) DNA substrates [42]. Besides their role in tethering and orienting the DNA substrate for subsequent cleavage, these homodimers possibly evolved to bind DNA in a conformation that simultaneously allows interactions with PCNA, FANCD2-FANCI and other proteins (Fig. 2c). It would be interesting to elucidate the contribution of the *endo*- rather than the *exo*-nuclease activity in the processing of damaged DNA, by employing dimer-defective FAN1 variants (K525E/R526E/K528E triple mutant or a 510–518 deletion) [41, 42]. Curiously, FAN1 dimerization is unique to higher eukaryotes [28, 41].

FAN1 was initially detected in a proteomic analysis of MLH1- and PMS2-interacting proteins, undertaken to gain novel insights into the function of MMR [4]. Individual interactions between FAN1 and MutL homologs were subsequently validated using reciprocal immunoprecipitations and size-exclusion chromatography [5, 7]. It is currently unknown whether complex formation between FAN1 and MutL α , MutL β , or MutL γ heterodimers is mediated via their common MLH1 subunit or via a common interaction motif in PMS2, PMS1 and MLH3. MutL-binding region(s) in FAN1 have not yet been mapped. MLH1 is not required for nuclease activities of FAN1 *in vitro* [6]. The FAN1-MLH1 interaction may play a structural role, which potentially determines DNA cleavage specificity. Finally, it has been proposed that FAN1 interacts with MSH2 following treatment with alkylating agents [43], but this finding has not been independently confirmed and direct interaction of FAN1 with MSH2 (or its partners MSH3 or MSH6) was not demonstrated.

3. FAN1 AND DNA REPAIR-RELATED PROCESSES

Loss of FAN1 results in sensitivity to drugs that induce DNA crosslinks (e.g., MMC, chlorambucil, and platinum-based drugs) [5–8, 44], albeit far less (by at least 30-fold) than with deficiencies of either FANCA, FANCI, or FANCD1 (XPF/ERCC1) [6, 45, 46]. Moreover, FAN1 depletion leads to only a very mild (<5-fold) increase in spontaneous and MMC-induced chromosomal aberrations (chromatid breaks, exchanges, chromosome-type aberrations, multi-radials), compared to those in FA cells (up to 100-fold) [6, 45]. ICLs can be processed either in S-phase by replication-coupled pathways that require the NEIL glycosylase [47] or FA proteins [48, 49], or in a replication-independent manner involving TLS or MMR pathways [50, 51]. Both replication-dependent and replication-independent ICL repair pathways share several enzymatic steps, which rely on the activity of structure-specific nucleases.

FAN1 can cleave ICL-stalled replication-like structures [29, 52] (Figs. 3 and 4a), indicating its role in S-phase-dependent ICL repair. There has been considerable effort to define the function of FAN1 in processing of DNA modified from endogenous or exogenous sources, but its precise role in ICL repair is still unclear. ICLs induced by exogenous compounds—such as cyclophosphamide, cisplatin, melphalan, psoralens, diepoxybutane, or MMC—have been used as proxies for the repair of endogenous ICLs caused by common metabolites, such as formaldehyde and acetaldehyde (including that derived from ingested alcohol), and oxidized lipid species [7, 53, 54]. FAN1 can resolve a subset of ICL-containing structures, including those where the replication forks remain some distance from the damage [52] (Figs. 3 and 4a), thereby generating a suitable substrate for subsequent nicking by a 3' flap endo-nuclease (such as XPF-ERCC1) and DNA synthesis by TLS polymerases (Fig. 4b). Biochemical evidence suggests that the primary function of FAN1 in ICL repair lies in its ability to 'unhook' the DNA lesion [55]. Although FAN1's nuclease activity is well suited for ICL incision *in vitro* [29], its depletion in *Xenopus* egg extracts does not lead to any incision defects [56]. This discrepancy might reflect a difference in the chemical nature of the lesions studied. In the replication-dependent ICL repair system of *Xenopus*, unhooking only occurs when replication forks are in very close proximity (at least 5 nucleotides)

to the crosslink: an unfavorable scenario for FAN1 [52, 56]. Alternatively, immune depletion of FAN1 in *Xenopus* egg extracts may not cause discernable defects in ICL incision because of redundancy with other structure-specific nucleases (for review, see [57]). Thus, in mice, Fan1 is non-epistatic to Slx1 [58]—a nuclease that can process crosslinks by promoting the endo-nucleolytic cleavage of a wide range of branched structures, including 5'-flaps (Fig. 3). However, while Slx1 processes ICL-containing structures at the DNA branch point [59], FAN1 can incise both the single-stranded DNA (ssDNA) flap, and double-stranded DNA (dsDNA) region downstream [7, 28, 41]. FAN1 and SLX1 may cleave distinct ICL-containing DNA sequences, or they may operate at different steps of ICL repair, with SLX1 playing a more prominent role in the unhooking step. FAN1-mediated cleavage of ICL-containing substrates *in vitro* is reminiscent of the activity of SNM1A, the human homolog of Pso2, a structure-specific nuclease involved in ICL repair in *S. pombe* [60]. To date, SNM1A and FAN1 are the only established nucleases that can traverse crosslinks and exhibit both 5'-flap endo-nuclease and 5'-3' exo-nuclease activities [53, 61]. Human SNM1A complements the ICL sensitivity in yeast that lack Pso2 [60], but its deficiency confers very mild crosslink sensitivity in mammalian cells [32], probably because of the redundancy with FAN1. Accordingly, yeast *Fan1* and *Pso2* are non-epistatic in terms of cisplatin sensitivity [40] and *Snm1a* partially compensates Fan1 loss in mouse cells [32].

In addition to a direct role in ICL unhooking, it has been speculated that FAN1 may act on HRR intermediates [6, 7] that form during the last step of the ICL repair process (for review, see [62]). HRR requires the formation of a DNA double-strand break (DSB), followed by DNA end resection by the MRN-RPA-BLM-DNA2-EXO1 machinery [63]. Then, the BRCA1-PALB2-BRCA2 complex [64] allows assembly of the RAD51 nucleofilament in order to promote a homology search in the intact sister chromatid. While FAN1 seems not to be required for DSB resection [7], its deficiency leads to a delay in the disappearance of RAD51 foci [6, 7], indicating a role in the late stage of HRR. Alternatively, ICLs can result in fork collapse, generating one-ended DSBs that require break-induced replication (BIR) (a subpathway of HRR) for fork restart (for review, see [62]). Thus, the lagging strand 5' to the ICL provides a potential substrate for FAN1 endo-nuclease activity, which can create a nick and further promote

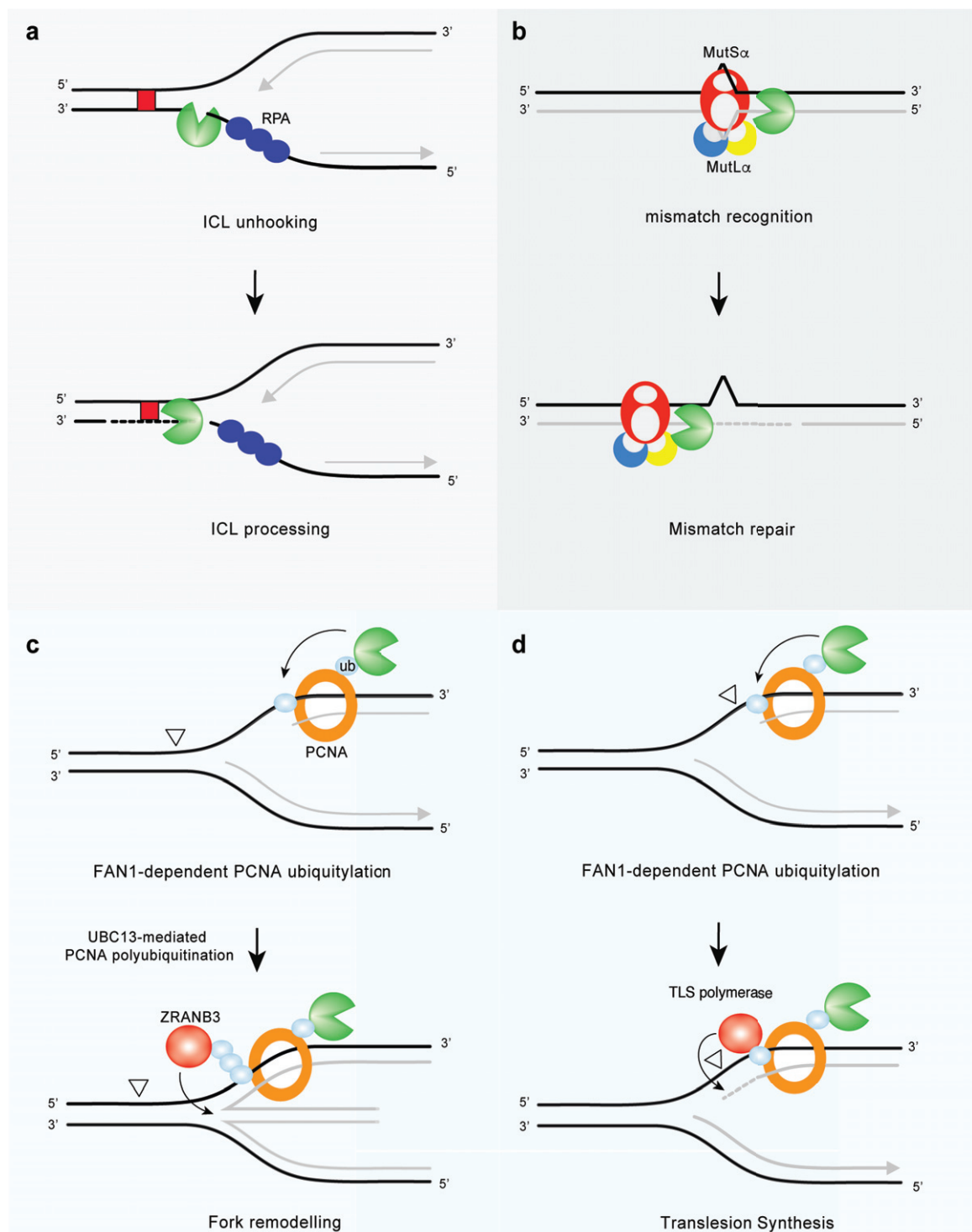


Fig. 4. FAN1 at the cross-road of genome maintenance pathways. (a) FAN1 promotes FA pathway-independent repair of ICLs. FAN1 catalyzes ICL unhooking by *endo*-nucleolytic incision followed by *exo*-nucleolytic digestion of DNA past the lesion, thereby generating a substrate for TLS polymerases (ICLs are indicated by red box). (b) FAN1 interacts with MutL α and may function as a compensatory nuclease in MMR in the absence of EXO1. (c) FAN1 promotes PCNA mono-ubiquitylation on the K164 residue. Further modification of the same residue via K63-linked polyubiquitination is then required for the recruitment of the ZRANB3 translocase and fork reversal, an error-free damage tolerance pathway. (d) FAN1 binds ubiquitylated PCNA on K164 and activates a feed forward loop that enhances PCNA ubiquitylation and possibly TLS (aberrant DNA lesions are indicated by open triangles).

the trimming of the nascent DNA. This generates a 3' ssDNA overhang, required for resumption of DNA synthesis [41, 52]. While FAN1 can cleave ICLs *in vitro*, its role in the cellular response to MMC or other crosslinking agents remains unknown. Therefore, it is of fundamental importance to functionally delineate FAN1-mediated ICL processing in cells and *in vivo*.

FAN1-mediated processing of damaged DNA relies on its accurate recruitment to the site of the lesion. Although FAN1 structure might be crucial for its substrate preference, scaffold proteins are required to secure its timely localization. Initially, FAN1 has been associated with the FA pathway because of its binding to ub-FANCD2—a central component of the FA machinery. The FAN1-FANCD2 interaction mediates FAN1 recruitment to sites of ICLs and has been thought to be crucial for ICL repair. This long-held belief is now challenged by the evidence that cells expressing the UBZ-mutated form of FAN1 are not hypersensitive to MMC [30, 32]. Thus, the contribution of the UBZ domain in FAN1 to ICL repair, if any, remains largely unknown. It may bypass the requirement of the FA proteins and become crucial for ICL repair in a context where the FA pathway is inactive. Along this line, FAN1 deficiency enhances ICL-induced genomic instability in *FANCD2* null cells and further sensitizes *FANCA*-deleted cells to MMC [32]. Chicken DT40 cells depleted of both FAN1 and FANCC are more sensitive than wild-type and single-knockout cells to cisplatin [44]. In addition, kinetics of FAN1 foci formation is largely unaffected in PD20 cells that lack FANCD2 [32]. Together, these findings suggest a role for FAN1 in ICL repair that is outside of the FA pathway, explaining why FAN1 mutations have not been identified in unclassified FA patients. The chemical nature of ICLs, the resulting DNA distortion, and the location of the damage will influence the mode of its repair. Thus, a role for FAN1 might be in the formation of a one-ended DSB, only for the subset of forks that do not physically encounter the cross-link and do not activate the FA core complex. Intriguingly, FAN1 appears earlier than the FA pathway on the evolutionary scale. A FAN1 homolog lacking a UBZ domain is indeed implicated in the repair of ICLs in unicellular eukaryotes, such as fission yeast (*S. pombe*), that do not possess the FA machinery [40]. We thus speculate that the FAN1-FANCD2 interaction is not directly related to the repair of DNA crosslinks, and that an as-yet unidentified factor is responsible for mobilizing FAN1 to sites of ICL in a UBZ-independent

manner. Localization of FAN1 at sites of ICL damage is biphasic, with an initial rapid recruitment of the protein followed by a slower but steady accumulation, which coincides with FANCD2 binding [32]. While the retention of FAN1 at ICLs depends on its UBZ domain and is not associated with ICL sensitivity, the first, very fast phase of this process apparently confers resistance to ICL-inducing drugs and relies on the FAN1 SAP domain [32]. However, it is very unlikely that the direct interaction of FAN1 with DNA can specifically drive the protein to ICL sites in a timely manner. Since the SAP domain is also involved in FAN1 dimerization, mutations in this module can perhaps perturb FAN1 dimer structure and rewire interaction networks, ultimately affecting FAN1 recruitment.

A direct role for FAN1 in post-replicative MMR seems apparent, based on its interaction with MutL homologs MLH1 and PMS2, together forming the MutL α heterodimer [4] (Fig. 2c). The MMR system detects and removes non-Watson-Crick base pairs in both mitotic and non-mitotic cells. The absence of MMR in states of high proliferation leads to high levels of mutations [65]. Thus, functional MMR is essential to ensure the fidelity of DNA replication but also for genome integrity in non-mitotic cells. Cells treated with the alkylating agent, N-methyl N-nitrosourea (MNU), accumulate FAN1 nuclear foci in an MLH1-dependent manner [43]. Authors of this study propose that FAN1 *exo*-nuclease generates ssDNA to activate a DNA damage response, ultimately triggering apoptosis [43]. To formally prove a role of FAN1 in MMR, one should monitor the rate of G:C-to-A:T transversion mutations in a FAN1-deficient background following MNU treatment. Independent studies reported that FAN1 inactivation causes no evident defect in MMR [5, 66] in normal settings, but that FAN1 may be one of several redundant MMR nucleases, which may include (but may not be limited to) EXO1, MRE11, Artemis, FEN1, and XPF [66]. Thus, FAN1-deficiency may contribute to a partial MMR defect only in certain genetic backgrounds.

MMR complexes are implicated in recognition and processing of ICLs and other forms of chemically damaged DNA, in addition to mismatched bases. In particular, MutS α (MSH2-MSH6) binds to ICL-containing plasmids and promotes recruitment of MutL α [51], whereas MutS β —which has higher specificity for larger insertion-deletion loops (IDLs)—associates with psoralen-induced ICLs [67]. Recent reports suggest a role for the MMR

machinery upstream of the FA pathway in the mono-ubiquitination and chromatin localization of FANCD2 [68]. Thus, it is conceivable that the FAN1-MLH1 complex might be recruited to sites of ICLs in S-phase at an early step that precedes FANCD2 localization. Future experiments should address whether FAN1-MLH1 interaction is required for the FAN1-dependent ICL repair, analogously to what has been proposed for the DNA helicase FANCDJ [69, 70]. In this respect, it will be crucial to test whether MutL α can act as a bridge allowing FAN1 nuclease to pair with the FANCDJ helicase during ICL processing.

4. FAN1 AND DNA REPLICATION STRESS

Replication stress is defined as perturbations in replication dynamics that alter fork progression. A number of DNA regions in the genome are intrinsically difficult to replicate and might represent a hurdle for the DNA replication machinery. For example, repetitive DNA sequences—such as ribosomal DNA, telomeres, GC-rich regions and expanded trinucleotide repeats—can assemble into secondary DNA structures that are thought to impede fork progression or cause replication slippage. Other DNA regions with low density of replication origins, such as common fragile sites, are prone to form gaps and stretches of ssDNA; thus, they are tied to replication stress. Heavily transcribed regions display elevated levels of stress because of increased probability of a clash between replication and transcription machineries. In addition to these endogenous sources, cells may experience various forms of exogenous DNA damage that perturb replication fork progression. Beyond its role in ICL repair, FAN1 contributes to DNA replication and is associated with replication stress responses (Fig. 4c, d). The first evidence linking FAN1 to replication fork processing implicates its nuclease activity in the restart of forks stalled by aphidicolin (APH), a specific inhibitor of B-family DNA polymerases [71]. At sites of APH-stalled replication forks, FAN1 associates with FANCD2 and the BLM helicase in a trimeric complex to promote fork recovery and suppresses new origin firing. In this context, the UBZ domain of FAN1 appears to be dispensable, whereas its chromatin localization requires non-ubiquitylated FANCD2 [71]. Upon fork stalling, FANCD2 targets the replisome by establishing a transient interaction with the replicative MCM helicase, independently of its mono-ubiquitylation

by the FA core complex [72]. Non-ubiquitylated FANCD2 may grant FAN1 access to the replication machinery and permit fork restart in a timely manner. In the absence of FANCD2, FAN1 can be still mobilized to stalled forks and drive extensive nucleolytic processing of nascent DNA strands [71]. Thus, the major role of the FAN1-BLM-FANCD2 trimeric complex may be to restrain FAN1 nuclease activity and ensure appropriate processing of stalled forks. In particular, FANCD2 might limit FAN1 activity at stalled forks by stabilizing RAD51-DNA filaments [73]. The role ascribed to FAN1 in fork metabolism is reminiscent of that attributed to DNA2—another nuclease involved in fork restart [74], which can mediate over-resection of nascent DNA strands if not kept in check [74, 75]. FAN1 localization at APH-stalled forks also relies on MRE11's 3'-5' exonuclease activity [71], which can reshape the fork into a suitable substrate for FAN1. Besides its role in fork restart, FAN1 nuclease activity is required to restrain replisome progression and preserve genomic integrity, under conditions of dNTP shortage due to hydroxyurea treatment [30]. Surprisingly, the UBZ domain, which is dispensable for FAN1-mediated fork restart, appears to be crucial for limiting fork progression and chromosomal instability following replication stress [30]. Likewise, the FAN1 interaction with ubiquitylated-PCNA is required to limit the rate of DNA synthesis under replicative stress conditions [34]. Further investigation is needed to establish how FAN1 can, at the same time, limit fork progression and promote fork restart. Like FAN1, the HLTFT translocase [76] or the RAD51 recombinase [77] can restrain DNA synthesis during replication stress by promoting fork remodeling—a process that converts replication forks into four-way structures and back. Replication fork reversal allows the replisome to bypass UV-damaged templates [78] and represents a pathway that allows DNA replication to pause and resume once the damage is repaired (for review, see [54]). Thus, it is tempting to speculate that FAN1-dependent processing of stalled forks might operate at the initial step of fork reversal by promoting localization of the SNF2 family DNA translocases—such as ZRANB3, HLTFT and/or SMARCAL1—whose activity has been involved in fork regression [79]. In particular, ZRANB3 fulfills its functions by binding poly-ubiquitylated PCNA. In response to replication stress, FAN1 activates a feed-forward loop able to enhance levels of mono-ubiquitylated PCNA, providing potential substrates for poly-ubiquitin chain extension. The latter will ultimately help ZRANB3 to

engage replication factories efficiently and promote regression of the stalled fork. Future research should aim to prove this model and determine whether FAN1 accomplishes its replicative function by promoting fork reversal (Fig. 4c). On the other hand, given that the formation of reversed forks places the DNA lesion back into the context of dsDNA, we cannot rule out a role for FAN1 in the incision of the region flanking the damage, analogous to its putative function in ICL repair.

Alternatively, FAN1 can channel stalled forks into the TLS pathway, allowing replication through a damaged template with low processivity and fidelity (Fig. 4d). FAN1 might indeed process forked DNA in a way that generates RPA-coated ssDNA: a pre-requisite for the recruitment of the E3 ligase, RAD18, and subsequent PCNA ubiquitylation [80]. Ubiquitylated -PCNA can enable mobilization of TLS DNA polymerases, which bypass bulky lesions in a post-replicative manner (Fig. 4d). The latter may account for the limited fork speed observed in FAN1-proficient cells upon replication stress induction. On the other hand, permissive replication detected in FAN1-deficient cells might be associated with extensive repriming and/or unscheduled origin firing. Both of these processes lead to an excess of ssDNA gaps that, if left unreplicated, might overwhelm the post-replicative repair mechanisms in G2, generating DSBs and chromosomal instability. Thus, a key objective for future research will be to investigate whether unrestrained replication in FAN1-deficient cells generates S1-nuclease-sensitive tracts, which would indicate ssDNA regions. Moreover, it will be important to explore the role of FAN1 in replisome surveillance in cells lacking either PrimPol—which has emerged as a key factor in re-priming [81]—or Cdc7 kinase activity, which otherwise stimulates dormant origin firing [82]. Such studies will help to define whether FAN1 may serve as a molecular switch to balance fork reversal, TLS, or re-priming events during replication stress.

5. *FAN1* DEFICIENCIES AND VARIANTS IN GENETIC DISEASES AND CANCER

Many *FAN1* variants have been associated with various diseases, including CAG/polyQ-diseases, karyomegalic interstitial nephritis (KIN), cancer, autism spectrum disorder, schizophrenia, epilepsy and Rett syndrome [14, 25, 31, 83–86] (Fig. 5). The relative contribution of FAN1 variants to each of the

associated phenotypes is unknown. The connection of *FAN1* to KIN is by far the strongest, possibly followed by the modifier association with HD. Although ranking the strength of the other disease associations with FAN1 is difficult, we have approximated them by the size of the arrows in Fig. 5. The weight of the data supporting these associations varies, as discussed below. These variants and associated clinical presentations are summarized in Table S1, with variants pertinent to this review highlighted in Fig. 1b and d. The mechanism by which any of these variants contribute to the various clinical presentations is unclear but discussed in the sections that follow.

5.1. *Karyomegalic interstitial nephritis (KIN; OMIM#614817)*

Bona fide autosomal recessive mutations in *FAN1* lead to KIN (Fig. 5), a rare hereditary genetic disorder that leads to chronic interstitial nephropathy, first described more than four decades ago [87–89]. To date, more than 50 cases have been reported (reviewed by Isnard et al. [90]). KIN usually presents as a slowly progressing chronic kidney disease leading to end stage renal dysfunction in early adulthood [91]. The analysis of renal biopsy specimens usually shows non-specific but severe chronic interstitial fibrosis and tubular changes, associated with non-specific glomerulosclerosis and vascular lesions. Genetic deficiencies of *FAN1* have been identified in individuals affected by KIN [31]. Parenchymatous organs, such as liver, and kidney, neuronal tissue, and reproductive tissues show high levels of FAN1 expression [31], thus explaining why FAN1 variants might cause KIN (Supplementary Table 1). The disorder is characterized by fibrosis of renal tubules; it differs from other forms of chronic kidney disease by virtue of the pronounced enlarged polyploid nuclei in the renal epithelium, rarely observed in other organs [92–95]. The source of the organ specificity of KIN is obscure. Kidney cells from KIN individuals showed >84% spontaneous hyperploidy; most were hyper-tetraploid 8-16C, but some had as many as 81 genomic equivalents [91, 96]. Consistently, *Fan1*-deficient mouse models recapitulate hallmarks of KIN pathology and also display liver function abnormalities, with hepatocytes showing increased ploidy [32, 58, 97]. Zhou et al. studied ten families with KIN [31] and identified 12 different mutations of *FAN1* in 9 of the families (Supplementary Table 1). Eight of the 12 mutations truncate the nuclease domain and three missense mutations (p.Q929P,

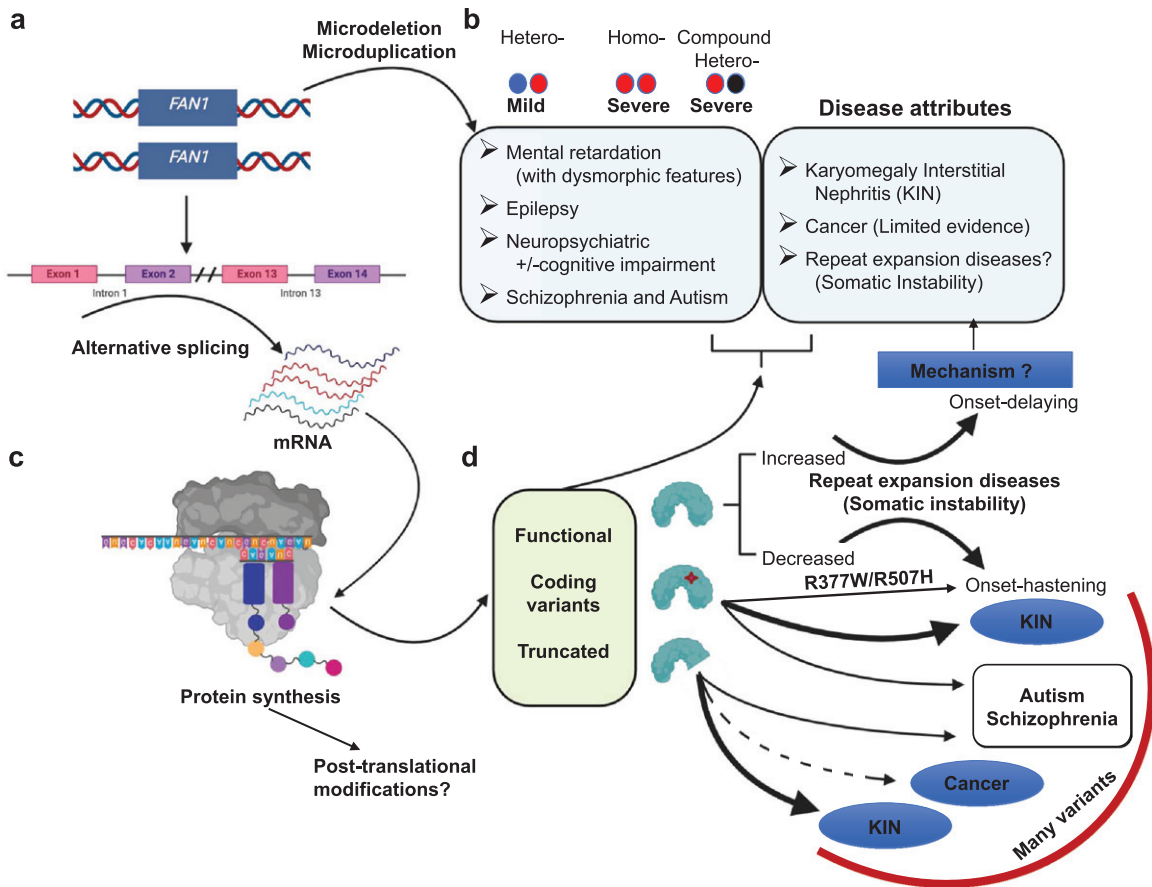


Fig. 5. Overview of *FAN1* genetics and its associated diseases. (a) Heterozygous microdeletion in the *FAN1* gene is associated with a mild form of 15q13.3 microdeletion syndrome. Whereas homozygous microdeletion or compound heterozygous microdeletion is associated with increased severity. Microduplications can be associated with various symptoms. (b) Defects in *FAN1* identified in various diseases (c). These defects may be due to alternative splicing. It may generate aberrant transcript, which further on translation produces either functional protein, coding variants, or truncated *FAN1* protein. (d) Various forms of *FAN1* proteins are identified in multiple diseases such as truncated protein and coding variants are identified in various cancers, KIN, schizophrenia, autism, and some disease hastening coding variants identified in HD and SCAs. The relative strength of these associations varies widely and are discussed in the text. Arrows that are bold, medium and dotted denotes strong, moderate and limited evidence for the disease association, respectively. The mechanism associated *FAN1* role with all of these diseases is unknown.

p.G937D and p.D960N) are in evolutionarily conserved amino acid residues of the *FAN1* nuclease domain [31]. They found none of these mutations in 96 healthy individuals. Consistently, mice expressing a truncated form of *Fan1*, devoid of its nuclease activity, develop a mild form of KIN [58]. Analysis of medical records showed that the p.R507H variant of *FAN1* associated with KIN in population cohorts, contributing in unknown ways [98]. It is notable that multiple individuals with homozygous 15q13.3 microdeletions encompassing *FAN1* do not show signs of KIN; rather they present with global developmental delay or intellectual disability, and severe hypotonia [23, 45, 99–106]. A major open

question in the pathophysiology of KIN concerns the nature of the lesions repaired by *FAN1*. Polyploidy (up to 8C) observed in *Fan1*-deficient mice increases during development or in response to ICL-inducing agents, but not to hydroxyurea [32, 58], suggesting that increased ploidy arises by a defect in ICL repair or in the processing of ICL-stalled replication forks. The increased ploidy might be a direct consequence of re-duplication of the whole genome or might reflect the presence of portions of the genome that are under-replicated. Either way, the appearance of polyploidy implicates replication cycles not followed by accurate mitosis. The ICL-induced polyploidy is independent of *FANCD2* [58].

5.2. Cancer

FAN1 deficiency or variants are associated with cancer (Supplementary Table 2), however, *FAN1* is not a FA gene *per se*. Individuals carrying *FAN1*-deficiencies do not present with the clinical attributes typical of FA patients (i.e., anemia, bone marrow failure, skin pigmentation anomalies, skeletal abnormalities of the upper limbs, or spontaneous leukemia) [45]. Aged mice carrying a defective Fan1 nuclease domain do eventually develop pulmonary carcinomas and hepatic lymphomas [30]. *Fan1*-deficient mice do not display discernable defects in bone marrow histology nor attrition of the hematopoietic stem cell compartment [30]. The p.M50R of *FAN1* increases risk of colorectal cancer (CRC) [107] and occurs as germline mutation in human hereditary pancreatic cancers [33]. Heterozygous germline *FAN1* mutations in MMR-proficient families suggest that it may be a CRC susceptibility gene [108]. A meta-analysis suggested that the contribution of *FAN1* to CRC is doubtful, as the evidence of segregation is weak ($p=0.125$), with no somatic mutations of *FAN1* in tumors from any of the *FAN1* mutation carriers [108, 109]. Consistent with this, another meta-analysis raises serious concerns about the role of *FAN1* in cancer progression and predisposition [85]. Among >5000 cancer patients clinically suspected as having genetic predisposition to colorectal, ovarian, breast, and other cancers, monoallelic pathogenic *FAN1* variants were identified in 0.35%. However, the expected frequency of heterozygous mutations in patients and the general population is also not significantly different [85]. No CRC was reported in the first- or second-degree relatives of any of the 16 index cases with pathogenic *FAN1* variants. No cancer was identified in close blood relatives of four index cases with KIN carrying biallelic germline mutations of *FAN1* nor in patients with homozygous 15q13.3 microdeletions encompassing *FAN1* [85, 104]. Larger CRC series could provide more information about the prevalence of *FAN1* pathogenic variants. A thorough analysis of genetic and genomic alterations found in *FAN1*-associated tumors will be important to clarify the underlying repair defects accumulated by these cells and mechanism of action of *FAN1* in colorectal carcinogenesis. Interestingly, *FAN1* may acquire a key role in cancer cells lacking BRCA2 [34]. BRCA2 is an important factor of HRR and acts as reservoir of the RAD51 recombinase [64, 110–113]. Deposition of RAD51 by BRCA2 not only facilitates HRR but has a fork-protective

function by preventing replication stress in a manner distinct from HRR [114]. *FAN1* limits replication stress and genome instability in BRCA2-deficient cells [34] and may provide a protective mechanism in cancer cells lacking BRCA2, ultimately affecting cell survival and treatment responses. In light of these data and resolution of the *FAN1* crystal structure, *FAN1* inhibitors could be developed for treatment of BRCA2-deficient tumors. Finally, increased *FAN1* expression correlates with poor prognosis of breast and ovarian cancers that are refractory to therapy [115]. Therefore, *FAN1* might be a novel promising target for cancer therapy or be used as potential biomarker to predict the insurgence of drug resistance in breast and ovarian cancer.

5.3. Neurodevelopmental and neuropsychiatric diseases

FAN1 copy number variations (CNVs), via deletions and duplications of the 15q13.3 region, are associated with autism, schizophrenia, and epilepsy [21, 25, 83, 84] (Fig. 5). These neurodevelopmental, psychiatric, and neurological disorders, respectively, share genetic predisposition factors [21, 25, 83, 116, 117]. Whole exome sequencing of autism patients and whole genome sequencing of schizophrenia patients identified various variants including *FAN1* missense variants associated with both autism and schizophrenia [25, 83, 84] (Supplementary Table 2). Likewise, recurrent *FAN1* CNVs predispose to an array of psychiatric and neurodevelopmental disorders [116, 117]. A homozygous knock-out mouse model involving the ortholog to a 1.5 Mb region of chromosome 15q13.3 spanning *Fan1* and seven adjacent genes displays strong phenotypes related to autism, schizophrenia, and epilepsy [21]. However, the degree to which these phenotypes are related to CNVs of *FAN1*, as opposed to any of the adjacent genes, is unknown.

Soon after the discovery that myotonic dystrophy and HD are caused by expanded tandem DNA repeat sequences, it was hypothesized that autism spectrum disorder (ASD), schizophrenia (SCZ), and epilepsy might also be caused by repeat expansions [118–120]. Indeed, various forms of epilepsy, SCZ, and—most recently—ASD are associated with repeat expansions [121–126]. Strikingly, Yuen and colleagues recently broadened, by leaps and bounds, the awareness of both the sequence motifs and the number of repeat loci that can be associated with a neurological phenotype [122]. *FMR1* CGG expansions cause a spectrum

of clinical presentations, including fragile X syndrome (FXS), autism, fragile X-associated ataxia, premature ovarian failure/insufficiency, attention-deficit disorder, learning disabilities, as well as psychological, endocrine, autoimmune, and metabolic disorders [127]. Other fragile sites caused by CGG expansions have also been associated with ASD. Ionita-Laza et al. reported numerous variants in *FAN1* as modifiers of ASD and SCZ [25], including p.R377W and p.R507H, which were subsequently identified as modifiers of onset in HD [14, 15]. The role of *FAN1* in any of these disorders and the underlying mechanisms by which it might mediate pathological effects need further investigation.

A coding variant of the murine *Fan1* is a modifier in Rett syndrome mice [86]—a neurodevelopmental autism-like disorder that occurs primarily in females. We feel that this finding is worth some attention for future studies in humans. Rett syndrome is caused predominantly by mutations in the Methyl-CpG-binding protein 2 (*MECP2*) gene; “atypical” Rett syndrome can result from variants in *FOXG1* or *CDKL5* [128–130]. A recent study screened for modifiers that improve subjective phenotypes in *Mecp2/Y* mice after mutagenesis with *N*-ethyl-*N*-nitrosourea [86]. Phenotypes assessed included limb clasping, tremors, body weight, activity, and longevity. Amongst several pathways, many of the identified modifier variants were in DNA damage response genes (*Birc6/BRUCE*, *Brca1*, *Brca2*, *Fan1*, *Mre11a*, *Rad50*, *Rbbp8/Ctip*, *Tet1*, and *Spin1*) [86] providing the first instance by which this mechanism may contribute to Rett syndrome. Absence of *MECP2* is associated with the accumulation of DNA damage [131]. The Rett-modifier in murine *Fan1* corresponds to a S798P change in human *FAN1* (Fig. 1), which is in between a highly conserved TPR and nuclease domain. How this *Fan1* variant modifies the *Mecp2*-deficient phenotypes, and whether such modifications extend to humans with Rett syndrome, requires further investigation.

6. GENETIC MODIFIERS OF CAG/POLYQ EXPANSION DISEASE ONSET

Certain neurodegenerative diseases are caused by the expansion of gene-specific CAG repeat sequences that encode polyglutamine (polyQ) tracts. Such diseases include HD (OMIM#143100), spinocerebellar ataxia type 1 (SCA1 OMIM#164400), SCA2 (OMIM#183090), SCA3 (OMIM#109150), SCA6

(OMIM#183086), SCA7 (OMIM#164500), SCA17 (OMIM#607136), and others. The age of onset of symptoms correlates with the length of the inherited CAG tract at the disease locus [132]. For the same inherited CAG size a broad range of onset age can occur, but less so within a given family [133], suggesting genetic modifiers of disease onset, independent of CAG tract size, must exist. Furthermore, Wexler et al. showed that 40% of the variance in age at onset in HD not attributable to CAG length, was heritable and therefore genetic [134].

The most robust evidence of DNA repair gene variants—including in *FAN1*—as modifiers of disease is for HD, where genome-wide analyses involve the greatest number of patients (HD cohorts of ~4000–9000), with strong clinical assessments (reviewed in [135]). The HD GWAS generated a statistical phenotype model, based upon HD subjects with 40–55 CAG repeats in the *HTT* gene in their blood DNA, to calculate the influence of the repeat length on age at onset of motor signs [15]. That study generated for each subject a “residual age at motor onset” value, which represents the difference in years between actual age at onset and that expected based upon the repeat size. These residual phenotype values were used to search the individuals genomes for genetic variations associated with HD age at onset, identifying tagSNPs that define the modifier haplotype.

6.1. *FAN1* variants in CAG/polyQ expansion diseases hasten or delay disease onset

DNA repair genes, *MLH1*, *PMS1*, *PMS2*, *MSH3*, *LIG1*, *RRM2B*, and *FAN1* act as modifiers of HD [14]. The strongest by far is *FAN1*, with both disease-hastening and disease-delaying tagSNP haplotypes [14,15] (Fig. 1b, d). Details about these HD modifier haplotypes are outlined here. For simplicity, we generally refer only to specific SNPs (rs#s), rather than to the haplotypes (please see Fig. 1d, and the review by Hong et al. [135]). Assessment of other diseases with CAG/polyQ expansion (SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17) also identified variants in *FAN1* as the strongest modifiers [136–138]. Those that act as modifiers of age of onset, the haplotype size, tagSNPs, genic location, and expected and documented effects, are summarized in Fig. 1d. Notably, some *FAN1* variants hastened or delayed disease onset by as much as ~6 years from that expected for the inherited CAG expansion size.

6.2. *Modifiers may alter expression levels of FAN1 mRNA*

The tagSNPs of *FAN1* associated with modifying HD onset may be associated with altered expression of *FAN1* mRNA, whereby increased or decreased FAN1 levels are beneficial or detrimental, respectively [12, 14, 15, 139]. Both expression quantitative trait loci (eQTL) and transcriptome-wide association studies [140] suggest that the SNPs are associated with changes of *FAN1* mRNA expression levels in specific brain regions but have no apparent effect on the expression of proximal genes, including *MTMR10* or *TRPM1* [12, 141]. In some individuals with HD, the onset-delaying *FAN1* modifier overrides the effect of the onset-hastening haplotype on the other *FAN1* allele [12]. These data support the concept that increased expression of FAN1 protein, and/or its functions, correlates with delayed onset and slower disease progression. For example, the tagSNPs rs35811129 and rs34017474 both correspond to *cis*-eQTLs for increased *FAN1* expression in cortex [12, 14, 141] and are associated with delayed age of onset. However, the 15AM4 haplotype associated with tagSNP rs34017474 does not capture single modifier effects; rather, a combination of the effects of the 15AM1, 15AM2, 15AM3 and 15AM5 haplotypes.

Expression studies provide a guide to how specific genetic variants might affect disease onset and progression but must be interpreted with caution. While the effects of *FAN1* variants upon its own mRNA expression in the cerebral cortex appear to correlate consistently with suspected increased or decreased levels of FAN1 protein, other factors must be considered. Notably, these *FAN1* expression studies use database (GTEx, CommonMind Consortium, and ROSMAP) from tissues of genetically defined individuals that were not affected with a CAG/polyQ disease [12, 14, 137, 141]. Individuals with HD have transcriptome-wide alterations, including in DNA repair genes, which must be accounted for [142, 143]. These dysregulated transcripts are evident in cell lines and patient tissues, and in many instances (but not all) are matched at the protein level [142, 143]. The size of the *HTT* repeat expansion can affect the degree of DNA repair gene dysregulation. For example, isogenic HD human pluripotent stem cells (hPSCs) with CAG lengths of 30, 45, 65, or 81 repeats, show increased expression of the DNA repair genes, *FAN1*, *PMS2* and *MSH3* at longer CAG repeat lengths [139]. Levels of the FAN1, PMS2,

and MSH3 proteins are significantly lower in HD brains compared to age-matched controls [139]. Such expression level variations may change as disease progresses. Tissue-specific expression effects can be important variables. For example, in different tissues, the rs3512 *FAN1* variant can have increasing or decreasing effects upon *FAN1* mRNA expression levels [137]. In FXS mice, expression levels of *Fan1* mRNA does not correlate to levels of Fan1-linked suppression of CGG expansions between the tissues [144]. Changes in mRNA expression levels do not always parallel changes in protein levels. None of the initial *FAN1* expression studies assessed FAN1 protein levels in any tissue [12, 14, 137, 141]. However, in human brains, FAN1 is decreased in the cortex of individuals with HD, while no significant difference is observed in the striatum [139]. Thus, haplotype-specific transcription level variations from non-HD databases are potent predictors of mechanism, but the expression level differences must be validated in the appropriate patient cells/tissues or experimental systems. Complexities of *FAN1* expression on CAG instability are discussed further in Section 7.2.

Limited data from HD patients support the concept that altered levels of FAN1 correlate with disease onset. As noted above, CNVs of the 15q13.3 region could increase or decrease the levels of *FAN1* and adjacent genes, which may affect disease. Some individuals with HD who carry three complete copies of *FAN1* (due to duplication of chr15:30900000-31500000) have significantly delayed onset compared to those with two allelic copies [12]. This finding supports the concept that increased FAN1 levels can be protective for HD; however, one cannot exclude the possible contribution of increased levels of other genes in the triplicated region, nor the presence or absence of any associated variants. It is important to distinguish the possible contribution of CNVs relative to any identified disease-modifying single nucleotide variants. This is particularly important in regions prone to CNVs, such as 15q13.3, which encompasses *FAN1*. CNVs are not responsible for the effects of individual single modifiers in the region [12].

6.3. *FAN1 coding variants, DNA binding, and nuclease activities*

Understanding the mechanism(s) by which the *FAN1* coding variants may alter disease is of great interest. Current thinking is that FAN1 may modulate repeat instability [12, 141]: a concept supported

by the hyper-instability of the expanded CGG repeat in somatic tissues of *Fan1*-deficient FXS mice [144]; however, biochemical evidence supporting this role is limited. So far there is no direct biochemical evidence of FAN1's role in the binding and processing of DNA repeats. FAN1 can recognize various secondary structures *in vitro*, such as chemically crosslinked DNA and DNA flaps [5–7, 29, 52] (Fig. 3). Two variants of *FAN1* that result in amino acid changes—p.R377W (rs151322829) and p.R507H (rs150393409)—are associated with hastening HD age of onset by 3.2 and 5 years, respectively [12, 14] (Fig. 1d). The p.R377W variant is proximal to Y374, which—along with Y436—contacts the DNA single-strand-duplex junction; these are required for appropriate DNA substrate orientation [29] but not DNA binding [41]. The missense mutation, Y374F, when coupled with Y436F, significantly impairs *endo*-nuclease activity on a 5' flap [41]. The HD onset-hastening p.R507H variant is located within the SAP-containing domain implicated in DNA binding [41]. One might speculate that the *FAN1* coding variants might affect the biochemical activities (DNA binding, protein dimerization, nuclease activity, etc.) of the FAN1 protein. The p.R377W and p.R507H FAN1 variants have decreased DNA binding to flapped-DNAs relative to that of the wild-type (wt) FAN1 [12]. Notably, this study used an indirect streptavidin-biotin-oligo pull-down method, with a crude nuclear extract from FAN1 knockout HEK293T cells over-expressing transfected FAN1 [12]. Binding constants were not calculated. The reduced DNA binding by the variants was evident whether the substrate contained a (CAG)10 loop-out or was devoid of repeats [12]. Binding activity for the FAN1 variants was assessed for only CAG (not CTG) slip-outs, and nuclease activity was not assessed. It is notable, that several nuclease-dead FAN1 mutants have DNA binding activities that do not reflect their absent nucleases activities. For example, the nuclease-dead mutant FAN1^{p.D960A}, retains DNA binding capacity comparable to FAN1^{p.wt}. In contrast, the FAN1^{p.K525E/R526E/K528E} shows undetectable DNA binding, undetectable *endo*-nuclease activity, but *exo*-nuclease activity comparable to wildtype FAN1 [6–8, 41].

FAN1 does not preferentially interact with the expanded CAG tract of the endogenous mutant *HTT* gene, as measured with chromatin immunoprecipitation (ChIP) [141]. No difference in FAN1 binding could be detected by ChIP between *FAN1* wt/wt or p.R507H/wt HD cells. FAN1 binding assessed by

ChIP at different genes with CAG, CTG, or CGG repeat tracts at other disease related genes (*TBP*, *ATXN3*, *DMPK*, or *FMRI*) is also not enriched at the expanded *HTT* CAG repeat nor any of the other non-expanded repeat genes. It is possible that ChIP is unable to detect subtle differences in DNA substrate preference, or between *FAN1* wt/wt and p.R507H/wt.

Nucleases can be classified as sequence-specific or structure-specific. DNA nucleases cleave the phosphodiester bond, yielding one cleavage product containing a 5'-terminal phosphate and one containing a 3'-terminal hydroxyl group. Nucleases can be further classified as *endo*-nucleases—which hydrolyse internal phosphodiester bonds—or *exo*-nucleases, which hydrolyse from the 5' or 3' ends of nucleic acids. Some nucleases are either *endo*- or *exo*- or can have both *endo*- and *exo*-nucleolytic activity. FAN1 is a structure-specific DNA nuclease with both *endo*- and 5' → 3' *exo*-nuclease activity.

The nuclease activity of FAN1 was reported as not necessary for FAN1's effect upon CAG/CTG instability [141]. To examine FAN1 function in repeat instability, FAN1 knockout U2OS cells with a stably integrated (CAG)118 construct, were reconstituted with FAN1^{p.wt}, nuclease-dead FAN1^{p.D960A}, or FAN1^{p.R507H} [141]. Repeat expansion was significantly slower in all FAN1 reconstituted lines compared to that in FAN1 knockout cells. There was no difference in CAG expansion rates between cells expressing the FAN1^{p.wt} and those with either the FAN1^{p.R507H} or the nuclease-dead FAN1^{p.D960A} mutant. Thus, neither the R507H nor the nuclease activity had any effect on CAG instability. Expression of the FAN1^{p.D960A} mutation on a p.R507H background did not affect CAG expansion rate relative to that of the catalytically active FAN1^{p.R507H}. These results led those authors to conclude that FAN1 “nuclease activity is not required for protection against CAG repeat expansion... *FAN1* affects somatic expansion of the CAG repeat through a nuclease-independent mechanism.” [141]. This is a counter-intuitive finding, as all FAN1 biological functions characterized to date depend on its nuclease activity [29, 32, 52, 97]. The exogenous repeat assay system and/or the induced overexpression of exogenous reconstituting FAN1 genes may not have been sensitive enough to “reveal” a subtle contribution from the nuclease activity. It is possible that the proficient DNA binding activity of the nuclease-dead FAN1^{p.D960A} [41] may, with its over-expression, in some way suppress CAG instability, as opposed to the expected enhanced CAG expansion observed in cells

lacking FAN1. Therefore, FAN1 nuclease activity could be more directly assessed via targeted mutation of the endogenous FAN1 nuclease domain in cells or an animal model.

One HD individual with a heterozygous rare mutation truncating the *FAN1* nuclease domain (rs184745027; p.R952*), had disease onset 11.7 years earlier than expected from CAG repeat length [12]. Biochemical experiments of FAN1 acting on repeat DNAs, additional cellular, and *in vivo* model system data are needed to determine the roles of the known FAN1 activities in modulating repeats and disease. It is worth noting that Exo1, like Fan1, suppresses CGG expansions, where Exo1 acts mainly in the gonads and Fan1 affects expansions in somatic tissues, but less so in the germline. Exo^{-/-} did not affect CAG instability in brain, liver, kidney, or heart, but did in intestine. In contrast, Fan1^{-/-} affected both CGG and CAG instability in each of those organs, but not in the germline transmitted lengths. Interestingly, the Exo1^{p.D173A} mutant, which abolishes its nuclease activity but retains its native conformation and DNA binding activity, shows a partial suppression of transmitted CGG expansions, intermediate between Exo1^{+/+} and Exo1^{-/-} animals [144]. Thus, in the mouse germline, this supports suppression of CGG expansions by Exo1 via both nuclease-dependent and nuclease-independent pathways.

7. FAN1 IN REPEAT INSTABILITY

7.1. Candidate nucleases in repeat instability; questionable involvement of Flap endonuclease 1 (*FEN1*)

There must be a DNA nuclease involved in the process of dynamic disease-associated repeat expansions and contractions; however, it is unclear which one is key. Candidates that have been tested include both *endo*- and *exo*-nucleases that recognize unusual DNA structures [145]. These include Rad27/Fen1/*FEN1*, DNA2, RecJ, Exo1/*EXO1*, SbcCD/Rad50/Mre11, Mus81-Eme1, APE1, GEN1, ERCC1-XPF, XPG, and WRN, which have been tested for a possible role in repeat processing or instability; *in vitro* assays and models range from bacteria, yeast, fruit flies, and mice, to human cells [146–193]. Considerable effort has been invested into *FEN1*, the Flap endo-nuclease 1 and its possible role in disease-associated repeat instability. In yeast, deficiencies of Rad27—the yeast *FEN1* homolog—increase CAG repeat expansions and contractions [149]. Yeast show a CAG contrac-

tion bias, in contrast to the expansion bias in humans. Transgenic mice and humans reveal no involvement of *FEN1* in CAG repeat instability [167, 188, 194]. Moreover, genetic variants of *FEN1*, known to be involved in cancer, show no disease association in families with either HD or Fuchs endothelial corneal dystrophy (FECD) (both CAG/CTG expansion diseases) [188, 194]. While it seems intriguing that *FEN1* can act upon flap-DNAs free of repeats, but its nuclease activity is inhibited by the presence of CAG/CTG hairpins, the relevance of this to repeat instability is unclear [160–162]. Until recently, there had been no human genetic evidence implicating involvement of any particular *exo*-nuclease. *FAN1* was the first *endo*-/*exo*-nuclease implicated in humans, as *FAN1* is the strongest modifier in genome-wide association studies searching for modifiers of age of onset for HD [14, 15].

7.2. *FAN1* variants modify disease by modifying repeat expansions

Attempts to decipher the role of *FAN1* and its variants in repeat instability have yielded mixed findings. One mechanism by which the coding *FAN1* variants might hasten onset of CAG/polyQ disease is through enhancing high levels of somatic repeat expansions. Initially, variants were tested for their impact on ICL repair/sensitivity. The effects of two coding *FAN1* HD-modifying variants, p.R377W and p.R507H, upon the function of *FAN1* are limited, and somewhat contradictory. A knockout of *FAN1* in HEK293T cells causes mildly increased sensitivity to MMC, which is rescued by transient expression of wt *FAN1*, but not by either variant form [12]. In contrast, the wt or the p.R507H *FAN1* rescued to comparable levels the increased MMC sensitivity in *FAN1*-deficient U2OS cells [141]. Lymphoblasts from HD individuals with p.R507H/wt *FAN1* show increased MMC sensitivity, compared to those with wt *FAN1* [12]; however, another report shows no such difference [141]. The contradictory results may reflect other genetic variations between individuals, the numbers of HD lines studied, or different model cell systems, using different experimental conditions. The link (or lack thereof) of *FAN1*'s roles in repeat instability and any of its many known functions, including ICL processing, is unknown.

There are other instances of seemingly contradictory findings for the effect of the *FAN1* variants. For example, the non-coding rs3512 *FAN1* variant is one

of the strongest modifiers delaying onset for HD, SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17 [15, 136–138], and would be predicted to be associated with decreased somatic CAG expansions. However, while rs3512 is associated with increased FAN1 expression in blood [137], increased CAG expansion rates were detected in blood cells of HD individuals with this variant [137]. The association of rs3512 with increased somatic CAG expansions is inconsistent with the suspected decrease in somatic CAG expansions by which this variant would mediate a later age of onset. However, it is possible that this variant acts upon repeat instability differently in brain than in blood.

Deciphering the mechanism by which any one DNA repair gene variant may act on HD age of onset and/or CAG instability, is likely to be complex. For example, the *FAN1* rs3512 variant was associated with delayed onset for HD, SCA2, and some, but not all SCA3 populations [138, 195]. The rs3512 variant in a cohort of Chinese SCA3 patients was not significant, a discrepancy attributed to the diverse allele frequencies between ethnicities [196]. Moreover, the effect of the rs3512 variant in another cohort of SCA3 affected individuals was modulated by the size of non-diseased CAG lengths of the *ATXN2* CAG repeat, whose repeat when expanded causes SCA2 [138]. In fact, mounting evidence suggests that the presence of intermediate non-diseased sized CAG tract lengths at other disease repeat expansion genes can significantly modify the age of onset for a repeat expansion at another disease loci in the same individual [138]. The age of onset in HD individuals with *HTT* CAG expansions is modulated by CAG repeat sizes in the normal range of three other repeat expansion disease loci, *ATXN3*, (SCA3) *CACNA1A* (SCA6) and *AR* (SBMA) [197]. These associations suggest an interaction of these genes/gene products, possibly in a shared pathway. Notably, mutant HTT protein was recently shown to interact with the ataxin-3 protein, encoded by *ATXN3*, in a DNA repair complex [198, 199]. Besides from the *ATXN2*-SCA3-*FAN1* rs3512 association, whether these non-disease CAG tract lengths affect other disease modifiers is unknown. Also, an onset-delaying *FAN1* modifier can effectively be cancelled by other mutations. For example, one HD individual homozygous for the onset-delaying *FAN1* modifier rs35811129, expected to over-express FAN1 also had a rare mutation truncating the nuclease domain (rs184745027; p.R952*) in one *FAN1* allele [12]. Rather than delayed disease onset, this individual had onset 11.7 years earlier than

expected by their inherited CAG repeat length. Thus, the heterozygous loss of FAN1 nuclease activity produced an onset-hastening effect, over-riding the effect homozygous onset-delaying modifier. Thus, modifiers within the same modifier gene, or in separate modifier genes may synergize or cancel each other out.

In *FAN1*-knock-out U2OS cells, CAG expansions in an *HTT* (CAG)118 construct were greater than in cells that had wt *FAN1* added [141]. However, as noted in Section 6.2, that system was unable to distinguish between the effect of wt *FAN1* and that of p.R507H, or the nuclease-dead, p.D960A, even with the latter on a p.R507H background [141]. Thus, the effect of either the HD-hastening variant, R507H, or the nuclease domain could not be discerned relative to CAG instability. In iPSCs derived from an HD individual with (CAG)120 in the endogenous *HTT* gene, the repeat tract expanded by ~1 CAG unit every 12.4 days, over ~60 days [141]. ShRNA-mediated knock-down of *FAN1* significantly increased this to ~1 CAG unit every 9 days [141]. Similar effects were observed when the iPSCs were differentiated to post-mitotic medium spiny neurons: the vulnerable cell type in HD. Similarly, knocking out *FAN1* in HD-derived iPSC cells with (CAG)72, using CRISPR/Cas9, lead to gradual increases in the modal repeat size by ~1-5 units over six months of cell culture [12]. These results support a role of FAN1 in slowing, but not arresting, the rate of spontaneous CAG expansions of the endogenous *HTT* expanded CAG repeat. Since FAN1 can affect both CGG and CAG repeat instability, this now poses the question as to whether FAN1 might act upon any of the more recently discovered repeat expansion loci, and upon how many different repeat sequence motifs FAN1 might act? [121, 122, 200, 201].

The mechanisms by which FAN1 may suppress CAG or CGG repeat instability are unclear. Models proposed [202, 203] include a) replication-associated repeat instability, b) repair-associated repeat instability, and c) transcription-associated (R-loop) repeat instability (Fig. 6). That FAN1 can affect CAG instability in post mitotic cells argues against a role of FAN1 in instability at replication forks. The common ground for all these models is aberrant DNA structures formed by the expanded sequence, called slipped-DNAs, as intermediates of the expanded mutations [202, 204, 205]. Unusual non-B-DNA structures can form *in vitro* and *in vivo* at each of the unstable repeats (reviewed in [205–210]). In addition, all models of repeat instability require nucleases to

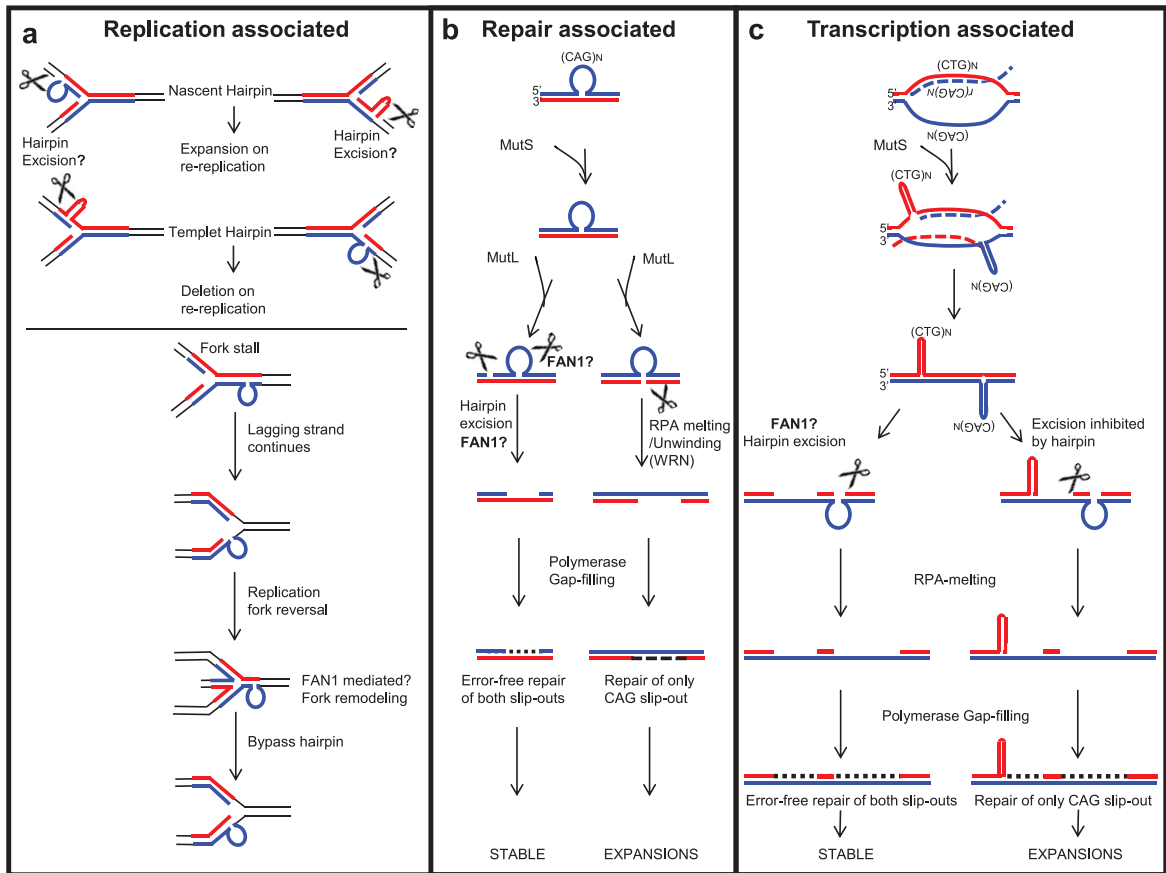


Fig. 6. Possible roles of FAN1 in processing repeat-containing DNA, protecting against repeat instability. (a) Replication-associated repeat instability; FAN1 may process slip-outs formed on a template or nascent strand during replication and may stabilize repeat length or induce contraction. (b) A slip-out formed by repeat-containing DNA can be processed by a non-mitotic DNA repair associated mechanism. FAN1 may act as one of the critical nucleases to process repeat slip-outs to stabilize the repeat tract against length variations. (c) In non-replicating cells, repeat instability is associated with active transcription across the expanded repeat. FAN1 may process the slip-outs formed following unidirectional or bidirectional transcription across the expanded repeat, to stabilize repeat tract against length variations.

process the slipped-DNA and thus protect the repeat tract from further expansion. Various DNA repair *endo*- and *exo*-nucleases have been studied with respect to repeat instability, such as FEN1, EXO1, MutL α and MutL γ [186, 202, 211–213]. Nonetheless, FAN1 is genetically implicated as a critical nuclease involved in repeat instability, yet the exact role of FAN1 nuclease in processing slipped-DNAs formed by repeat tracts is still unexplored (see Section 4.4 for details).

7.3. Role of FAN1 in replication-associated repeat instability

The replication-associated repeat instability model [202, 214–216] suggests that during replication of repeat-containing DNA, slip-out may form on

nascent or template DNA [202]. The slip-out on nascent DNA may lead to expansions by re-replication, whereas that on template DNA leads to contraction [217, 218] (Fig. 6a). Possibly an unknown nuclease processes the slip-out formed on nascent DNA, protecting it from expansion. With both *endo*- and *exo*-nuclease activity on structured DNA, FAN1 could perhaps fill this role.

The slip-out formed on the template strand may cause replication machinery to pause and allow dissociation of the replisome from the replication fork [202, 219, 220]. However, the lagging strand continuously synthesizes DNA, resulting in fork reversal (Fig. 6a). FAN1 may be involved in the TLS pathway and may process stalled replication forks (discussed in Section 3), protecting it from repeat instability. The recruitment of FAN1 to slipped-DNA following fork stalling has yet to be proven.

7.4. Role of FAN1 in repair-associated repeat instability

In HD patients, tissue-specific expansion-biased repeat instability is observed mainly in the brain and liver [154, 221]. However, brain neuronal cells do not undergo active replication, suggesting that somatic repeat instability is not limited to DNA replication, but may be induced by DNA repair mechanisms. Expression of FAN1 is high in brain cells, supporting its possible role in protecting against repeat instability. It is an interacting partner of MMR proteins, notably MLH1 [4, 5, 7, 34, 43], suggesting that it may be involved in MMR. Knowing how disruption of the FAN1-MLH1 interaction affects the severity of repeat expansion could be relevant to prediction of the onset of neurological disorders.

The requirement of MMR complexes—including MutS α (MSH2-MSH3), MutS β (MSH2-MSH6), MutL α (MLH1-PMS2) and MutL γ (MLH1-MLH3)—in CAG/CTG, CGG/CCG, and GAA/TTC repeat instability has been extensively studied [185, 210, 213, 222–229]. MutS β (MSH2-MSH3), through its ability to bind slip-DNA structures, may facilitate their formation [210]. MutS β recruits MutL complexes (Fig. 6b). MutL α has *endo*-nuclease activity on both the slipped-out and non-slipped-out strands, in unnicked DNA [186, 230]. In contrast, MutL γ *endo*-nuclease cleaves only the non-slipped-out strand [186]. MutL α or MutL γ generate a nick in the slip-out, potentially serving as an entry point for *exo*-nucleases like EXO1 or FAN1, both known to interact with MLH1 [231–233]. Any of these proteins may subsequently process the slip-out and stabilize the repeat expansion. In contrast, cleavage on the non-slip-out strand by MutL γ may lead to WRN-RPA-mediated melting of hairpin DNA [234]. Resulting gaps in the DNA may be filled by DNA polymerase β , which may lead to repeat expansion [166, 186, 234, 235] (Fig. 6b). FAN1 co-localizes with RPA at stalled replication forks in cells [236] and the nuclease activity of FAN1 is enhanced by RPA [237], but a direct interaction was not tested.

7.5. Role of FAN1 in transcription-associated repeat instability

In non-replicating cells, repeat instability can be driven by active transcription through the expanded repeat. Transcription of repeat-containing DNA leads to the formation of DNA:RNA hybrids, commonly

referred to as ‘R-loops’ (Fig. 6c). Slip-out structures may then form on the non-transcribed strand. Bidirectional transcription may lead to the formation of slip-outs in both strands [203, 238–241], which are recognized by MutS β and activate MutL complexes. As with repair-associated repeat stability, the relative contribution of MutL α or MutL γ will determine the fate of the slip-out, giving rise to either expansions, contractions, or stability. Repair of only non-transcribing slip-outs may result in repeat expansions, whereas repair of both slip-outs may lead to repeat stability [203, 210, 238–241] (Fig. 6c). However, for repair to happen, the slipped-DNA is processed by DNA nucleases: an aspect that needs to be further explored. In summary, it is possible that FAN1, through its *endo*- and/or *exo*-nuclease activity, is involved in the processing of these slip-outs, thereby protecting against further repeat expansion.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

FAN1 was initially thought to be associated with Fanconi anemia (FA); however, rather than FA, homozygous loss of FAN1 causes karyomegalic interstitial nephritis (KIN) [31, 45]. Most KIN mutations ablate nuclease activity of the enzyme, indicating that this activity protects normal kidney function [31]. Nuclease-defective FAN1 causes mild hypersensitivity to DNA crosslinking agents in cells and in mice, suggesting defects in ICL processing [31]. FAN1 can process ICLs in an FA-independent manner [29]. Its function in ICL repair and replication fork-protection might be responsible for safeguarding cells from endogenous and exogenous insults, including platinum-based and mitomycin C-based anti-cancer therapies. Loss of FAN1 function does not increase the burden of human cancers [85]. Considering that some cytotoxic anticancer agents act by inducing ICLs and/or replication stress, FAN1 nuclease activity is likely a good target for sensitization of cancer cells to killing by conventional chemotherapy. However, this might not be applicable to HD subjects who display lower cancer incidence than age- and sex-matched controls [242].

FAN1 is the strongest modifier in HD [14, 15]. Increased FAN1 expression and DNA binding may protect against somatic repeat expansion of trinucleotide repeats [12, 14, 141]. However, the full set of FAN1 functions—including the biochemistry of its DNA binding activity, dimerization, nuclease activ-

ities, various interaction partners, and roles in DNA replication and repair—need further investigation, particularly with regard to repeat DNAs. How this diverse set of DNA metabolizing functions may contribute to FAN1's involvement in multiple diseases is now a key focus. Variants in *FAN1* contribute to numerous diseases (including autism, schizophrenia, and epilepsy), some of which are caused by expanded repeat DNAs [20, 25, 122, 200, 201]. Current genetic analysis supports a role of ongoing somatic repeat expansions, play a role in the age-of-onset and progression rate of many such diseases. Regulation of CAG/CTG and CGG/CCG repeats by FAN1 opens the possibility that it may be involved with other unstable repeat motifs associated with disorders such as autism or schizophrenia [121, 122, 200, 201]. A focus on elucidating the mechanistic role of FAN1 in repeat instability is warranted, to learn if and how it modifies disease by modulating somatic repeat instability.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

SUPPLEMENTARY MATERIAL

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